Plant Biotechnology 28, 231–238 (2011)
DOI: 10.5511/plantbiotechnology.11.0106b

Original Paper

Isolation of cDNAs encoding tetrahydroxychalcone 2′-glucosyltransferase activity from carnation, cyclamen, and catharanthus

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Received October 1, 2010; accepted January 6, 2011 (Edited by M. Nishihara)

Abstract 4,2′,4′,6′-Tetrahydroxychalcone (THC) 2′-glucoside that confers yellow color to the petals of carnation, cyclamen, and catharanthus is biosynthesized by the action of UDP-glucose-dependent THC 2′-glucosyltransferase (THC2′GT). We isolated 18 types of full-length cDNA encoding GT-like sequences from carnation petals. Expression of these cDNA in *Escherichia coli* identified five cDNAs encoding THC2′GT that were different from the previously isolated THC2′GT. We also isolated a cDNA encoding THC2′GT from both catharanthus and cyclamen. These THC2′GT cDNAs were introduced to petunia. Transgenic petunia that expressed three of the GTs produced THC 2′-glucoside, which indicated that they function as THC2′GT *in vivo*. These cDNAs could be useful molecular tools to yield yellow flower color, although the amount accumulated in the transgenic petals was too small to alter the flower color in this study.

Key words: Carnation, chalcone, cyclamen, flavonoid, glucosyltransferase.

Flavonoids are one of the major secondary metabolites of plants. Flavonoids and their colored class, anthocyanins, are dominant constituents of flower color. Floral flavonoids are usually modified with one or more sugar moieties, and are localized in vacuoles (Tanaka et al. 2008). The glycosylation of flavonoids is catalyzed by family 1 glycosyltransferases of the 91 subfamilies of glycosyltransferases classified on the basis of sequence similarity, catalytic mechanisms, and the presence of conserved sequence motifs (Yonekura-Sakakibara 2009). Family 1 glycosyltransferases utilize specific UDP-sugars, such as UDP-glucose, UDP-rhamnose, and UDP-galactose, as sugar donors. Glucosyltransferase (GT) that utilizes UDP-glucose is the most common in this family. Family 1 glycosyltransferases contain a carboxyl-terminal conserved sequence, the secondary product glycosyltransferase box of plants (Gachon et al. 2005), and are classified into many clusters depending on sequence homology. Glycosyltransferases from different plant species having the same function usually belong to the same clusters in the family.

Glycosylation of flavonoids has been extensively studied in terms of its biochemistry and molecular biology. More recently, *Arabidopsis* flavonoid glycosyltransferases have been comprehensively analyzed (Yonekura-Sakakibara 2009). Flavonoid glycosylation, typically glucosylation, is catalyzed by position-specific glycosyltransferases. GT in the flavonoid biosynthetic pathway includes flavonoid 3-glucosyltransferase (3GT), flavonoid 5-glucosyltransferase (5GT), flavonoid 7-glucosyltransferase (7GT), and flavonoid 3′-glucosyltransferase (3′GT); 3GT and 5GT form separate clusters, and 7GT and 3′GT belong to the same cluster (Fukuchi-Mizutani et al. 2003; Tanaka et al. 2008). Acyl-glucose-dependent glycoside hydrolase-like anthocyanin GTs have been recently identified in carnations and delphiniums (Matsuba et al. 2010).

Chalcones and aurones are yellow flavonoids. 4,2′,4′,6′-Tetrahydroxy chalcone (THC) imparts pale yellow color but is rapidly isomerized to colorless naringenin by the catalysis of chalcone isomerase (CHI) *in vivo* (Figure 1), and spontaneously *in vitro*. THC color

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Abbreviations: AS, aureusidin synthase; CHI, chalcone isomerase; DFR, dihydroflavonol 4-reductase; EST, expression sequence tag; F3H, flavanone 3-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; GT, glucosyltransferase; RT, reverse transcription; THC, 4,2′,4′,6′-tetrahydroxychalcone; PKR, polyketide reductase.
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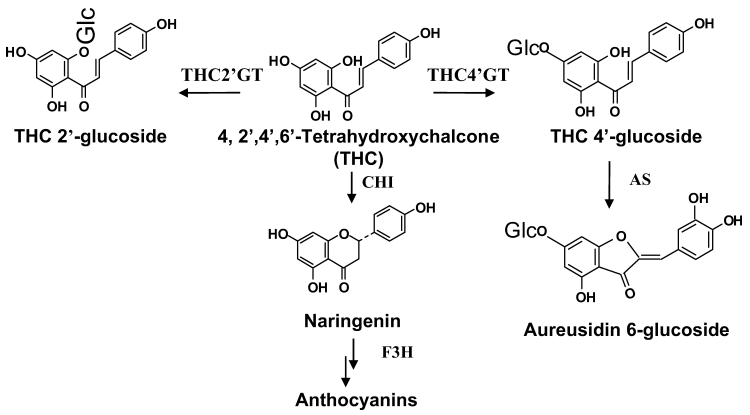

Figure 1. A part of flavonoid biosynthetic pathway relevant to THC glucosylation. GT, glucosyltransferase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; AS, aureusidin synthase; Glc, glucose.

is stabilized by 2′-glucosylation in the petals of carnation (*Dianthus caryophyllus*), cyclamen (*Cyclamen persicum*), catharanthus (*Catharanthus roseus*), China aster (*Callistephus chinensis*), and others. China aster and cyclamen that accumulate THC 2′-glucoside do not have CHI activity as reported by Kuhn et al. (1978) and Miyajima et al. (1991), respectively. Transposons are inserted into both *CHI* and *dihydroflavonol 4-reductase* (*DFR*) genes in carnations that accumulate THC 2′-glucoside. The transposon insertion inhibits anthocyanin biosynthesis by which the yellow color of THC 2′-glucoside becomes evident on carnation petals (Itoh et al. 2002).

It is rare for a single species to display all flower colors. It is possible to yield new color cultivars by expressing heterologous genes using genetic engineering (Nishihara and Nakatuska 2010; Tanaka et al. 2009). Transgenic carnations and roses wearing violet or blue hues have been generated by expressing heterologous *flavonoid 3′, 5′-hydroxylase* (*F3′5′H*) genes (Katsumoto et al. 2007; Tanaka et al. 2009). Some important floricultural crops, including pelargonium (*Pelargonium graveolens*), begonia (*Begonia semperflorens*), impatiens (*Impatiens walleriana*), and the Japanese morning glory (*Ipomoea nil*), lack yellow-colored varieties. Yellow cultivars of these species would offer high marketability.

Efforts to yield yellow color by genetic engineering have been partly successful. Reduction of THC to 4,2′,4′-trihydroxychalcone inhibits the cyclization and results in buteins, a type of chalcones. The reaction is catalyzed by polyketide reductase (PKR). Transgenic petunia plants wearing a very pale yellow color have been made by expressing *Medicago sativa* (Davies et al. 1998) or licorice *PKR* gene (Tanaka et al. 2005). Pale yellow cyclamen has also been made by inducing the expression of the *M. sativa PKR* gene (Mizukami et al. 2004). Aurone glucosides (aureusidin and bracteatin glucoside) conferring a more intense yellow color than chalcones are derived from THC 4′-glucoside by the action of aureusidin synthase (AS; Nakayama et al. 2000). Transgenic torenia plants accumulating aurones and wearing yellow color have been made by overexpression of *THC4′-glucosyltrasferase* (*THC4′GT*) and *AS* genes, and downregulation of anthocyanin biosynthesis by suppressing *flavanone 3-hydroxylase* (*F3H*) or *DFR* gene (Ono et al. 2006). However, the transgenic torenia plant accumulating aurone was shown to have lost the yellow color and exhibited growth retardation in a field trial (Tanaka et al. 2010). *THC2′GT* genes can be alternative and useful molecular tools to produce yellow color by genetic engineering. Ogata et al. (2004) isolated 18 types of *GT* genes from yellow carnations and identified two genes (*DicGT4* and *DicGT5*) encoding THC2′GT activity and three genes (*DicGT1*, *DicGT2*, and *DicGT3*) encoding anthocyanidin 3GT.

In this study, we isolated five cDNAs encoding THC2′GT activity from carnations; the genes isolated are different from those previously reported *DicGT4* and *DicGT5* (Ogata et al. 2004). We also isolated a cDNA encoding THC2′GT activity from both cyclamen and catharanthus.

Materials and methods

Materials

Yellow carnation (cultivar Light Cream Candle), yellow catharanthus (cultivar Yellow Magic), and pale yellow and pink cyclamens (normal type, unknown cultivars) were purchased from a florist. *Petunia×hydrida* line Skr4×Sw63 (Holton et al. 1993) and cultivar Baccara Red (Sakata Seed Co., Yokohama, Japan) were maintained in tissue culture for genetic transformation. Skr4×Sw63 showed pale pink flowers accumulating dihydrokaempferol due to deficiency of *flavonoid 3′-hydroxylase* and *F3′5′H* loci. Baccara Red showed intense red flowers accumulating cyanidin-based anthocyanins. THC 2′-glucoside and THC 4′-glucoside were purified from yellow carnation and snapdragon petals, respectively.

Isolation of GT homologs from carnation, cyclamen, and catharanthus petal cDNA libraries

General molecular biological procedures have been previously described (Fukuchi-Mizutani et al. 2003). Total RNA was isolated from the petals of yellow carnation buds at the stage when the tips of petals appeared from the buds. Poly A^+ RNA was prepared from the total RNA and subjected to directional cDNA library construction using Uni-ZAP XR (Agilent Technologies, Santa Clara, USA) as the vector.

Expression sequence tag (EST) of the cDNA library: DNA sequences of about 2,600 clones randomly selected from the cDNA library were determined from their 5′-ends using ABI 3700 DNA sequencer (Life Technologies, Carlsbad, USA). The sequences obtained were clustered, and subjected to BLAST search to identify GT homologs.

Sequence analysis of a subtraction library: Petal specific cDNA was prepared by subtracting leaf cDNA from the petal cDNA using Clonetech PCR-select cDNA subtraction kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer's protocol. The obtained cDNA was cloned into pCR-TOPO (Life Technologies). About 3,700 clones were subjected to DNA sequencing from both ends. BLAST analysis of the obtained sequences helped identify GT homologs, which were subsequently utilized as the probes to screen the petal cDNA library for the full-length clones. Hybridization was carried out overnight in 5×SSC containing 30% formamide, 0.1 M Tris-HCl pH 7.5, and 1% SDS at 37°C followed by washing in 5×SSC containing 1% SDS at 50°C for 1 h.

Library screening using *GT* genes involved in flavonoid biosynthesis: GTs have conserved regions in their C-terminal portions (Gachon et al. 2005). The corresponding DNA fragments were isolated from the cDNA of *I. nil* anthocyanidin 3GT (Morita et al. 2005), *Gentiana triflora* anthocyanidin 3GT (Tanaka et al. 1996), *Verbena×hybrida* anthocyanin 5GT (Yamazaki et al. 1999), and *Scutellaria baicalensis* flavone 7GT (Hirotani et al. 2000) with a few sets of DNA primers (InF: 5′-GAAATGGTCGGATTGGCTGGG-3′ and InR: 5′-ACCTCCACCCCAACTTTCAGG-3′, GtF: 5′-TGCCTCAAA-TGGCTTCAAACT-3′ and GtR: 5′-CCACCTTTCACCCC-AACCCC-3′, VhF: 5′-TGCCTCGAATGGTTGAGCACG-3′ and VhR: 5′-CTCTCACTCTCACACCCG-3′, and SbF: 5′-CACGAATGCTTAGCATGGCTC-3′ and SbR: 5′-CTTATTG-CCCACTGAAACCCC-3′). The DNA fragments were labeled with DIG and the mixture of the labeled DNA fragments was used as a molecular probe for screening the carnation petal cDNA library. Hybridization was carried out as mentioned above. The plaques that hybridized with the probe mixture were purified, and the plasmids recovered from the plaques were subjected to DNA sequence analysis.

Library screening with clone *T170* encoding THC2′GT activity: The carnation petal cDNA library was screened with *T170* cDNA as the probe as described above. Cyclamens (yellow and pink) and catharanthus directional petal cDNA libraries were constructed with Uni-ZAP XR as the vector. The constructed libraries were screened with the carnation *T170* cDNA as the probe under the same condition.

Measurement of THC2′GT activity of recombinant GT

Isolated GT cDNAs were inserted into pQE30 (Qiagen Inc, Valencia, USA), and then expressed in *Escherichia coli.* The expression of a *GT* gene in *E. coli* was induced by 20 μM IPTG at 27°C overnight in M9 medium supplemented with 0.5% Casamino acid. Harvested cells were treated with sonication and then centrifuged. The supernatant was subjected to THCGT assay as described previously (Fukuchi-Mizutani et al. 2003; Tanaka et al. 1996). Both soluble and insoluble fractions of *E. coli* crude extracts were subjected to SDS-PAGE followed by protein staining with Coomassie Brilliant Blue to detect a recombinant GT protein band.

THC was immobilized on a resin to prevent its spontaneous isomerization to naringenin. THC (500 μg ml^{-1} of ethanol) was mixed with 1 ml of Toyopearl HW-40F (Tosoh Co., Tokyo, Japan) and 3 ml of water. The mixture was used as a substrate for THC GT assay. The reaction mixture consisted of 100 μl of the substrate solution, 200 μl of crude extract, and 10 μl of 5 mM UDP-glucose. The reaction mixture was maintained at 30°C for 2 h, after which the resin was recovered by centrifugation, washed with water, and mixed with 300 μl of 50% acetonitrile containing 0.1% TFA. The mixture was treated with sonication to recover reaction products and THC from the resin.

The recovered flavonoids were subjected to HPLC analysis using a YMC-ODS-A312 column (6 mm×150 mm, YMC Co., Ltd., Kyoto, Japan). The elution profile consisted of a linear gradient from A : B=85 : 15 to 60 : 40 for 15 min (A is 2% acetic acid and B is methanol), A : B=60 : 40 for 5 min, a liner gradient to A : B=38: 62 for 10 min, and A : B=38 : 62 for 2 min. The flow rate was 1 ml min^{-1}. The elution was monitored with the absorbance set at 360 nm, and the absorption spectra of 250– 400 nm using a photo diode array detector (SPD-M6A, Shimadzu, Kyoto, Japan).

Generation of transgenic plants and their flavonoid analysis

Binary vectors expressing *GT* cDNA were constructed as described previously (Fukuchi-Mizutani et al. 2003; Ono et al. 2006) using *Mac1* (Comai et al. 1990) as the promoter for transcription of *GT* cDNA and pBinPlus (van Engelen et al. 1995) as the backbone vector. A transcriptional cassette to transcribe double-stranded petunia *CHI* (DNA data base accession number X14589) and/or petunia *F3H* (X60512) cDNA was inserted into the same vector in order to downregulate anthocyanin biosynthesis. The double-stranded RNA was transcribed by an enhanced cauliflower mosaic virus 35S promoter (Mitsuhara et al. 1996).

Petunia (Skr4×Sw63 or Baccara Red) was transformed with *Agrobacterium tumefaciens* Agl0 (Lazo et al. 1991) harboring a constructed vector. Total RNA prepared from the petals of the transgenic petunia was subjected to reverse transcription (RT)-PCR analysis to detect the transcripts of the introduced *GT* genes using SuperScript (Life Technologies) for RT followed by PCR, which consisted of 25 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min.

Petals (0.5 g) of the host and transgenic plants were extracted with 2.5 ml of 50% acetonitrile containing 0.1% TFA. The extract was subjected to HPLC analysis. To obtain better separation of endogenous substances and THC glucosides, plant flavonoids were separated with a gentler gradient; A : B=85 : 15 to 60 : 40 for 22 min (A is 2% acetic acid and B is

methanol), A : B = 60 : 40 for 5 min, a liner gradient to A : B = 38 : 62 for 14 min, and A : B = 38 : 62 for 2 min.

Results and discussion

Isolation of GT cDNA from carnation, cyclamen, and catharanthus petals and their sequence analysis

EST analysis of the carnation petal cDNA library yielded two kinds of GT homologs, *contig 232* and *L21C12*. *Contig 232* putatively contained full-length cDNA. Sequence analysis of the subtracted library resulted in 17 types of GT homologs, and one of them—*S1G1*—was identical to *contig 232*. Library screening with these 16 GT homologs yielded five types of full-length *GT* sequences (*S10B5*, *S6B11*, *S12A2*, *S6D11*, and *S22F3*). Library screening of a quarter million plaques with the mixture of conserved regions of flavonoid *GT* cDNAs yielded an additional 11 *GT* homologs, seven of which (*T11*, *T48*, *T170*, *T172*, *T181*, *T206*, and *T227*) were full-length. Clone *T128* was a full-length sibling of a non-full-length clone (*S27D3*) obtained from the subtracted library. Screening of the carnation library with *T170* yielded four more full-length *GT* homologs (*A14*, *A54*, *A82*, and *A93*).

The isolated *GT* genes from carnations are presented in Table 1. Amino acid sequences of S1G1 and T48 agreed with those of DicGT1 and DicGT3 (Ogata et al. 2004), respectively. A93 was longer than DicGT2 (Ogata et al. 2004) by about 80 amino acid residues toward the N-terminus and the rest exhibited 97% amino acid sequence identical to DicGT2. The phylogenetic tree (Figure 2), consisting of GTs isolated in this study and various GTs involved in the glucosylation of secondary metabolites, shows that the isolated carnation GTs are classified into various GT clusters. These results confirm that a plant species contains structurally versatile GT as reviewed for *Arabidopsis* (Yonekura-Sakakibara 2009). The experimental procedures adopted in this study are useful for isolating different varieties of *GT* genes from plants.

Screening of the cDNA libraries of yellow cyclamen, pink cyclamen, and catharanthus with *T170* resulted in five (*CpYCy3-12*, *11*, *17*, *31*, and *87*), one (*CpPCy1-12*), and one (*CrYMb4*) full-length GT homologs, respectively (Table 1).

Table 1 Summary of isolated GT homologs and their activity

Clone	Accession number	Nomenclature	Expression in *E. coli*	*In vitro* activity
Carnation				
Full-length cDNAs derived from cDNA library screening with T170 cDNA				
A14	AB294377	UGT84A8		Not detected
A54	AB294380	UGT71F5		Not detected
A82	AB294379	UGT75L1		Not detected
A93*	AB294378	UGT71F4		THC2′GT>THC4′GT
Full-length cDNAs derived from the sequence analysis of subtracted cDNA library				
S1G1	AB294392	UGT78E1		Not detected
S6B11*	AB294390	UGT73A7		THC4′GT>THC2′GT
S6D11	AB294389	UGT85M1		Not detected
S10B5 (T69)	AB294394	UGT73M1	ppt	Not detected
S12A2 (T112)	AB294393	UGT73A6		THC4′GT>THC2′GT
S22F3	AB294391	UGT79B15	ppt	Not detected
Full-length cDNA derived from cDNA library screening with conserved regions of GTs				
T11	AB294388	UGT86A3	ppt	Not detected
T48	AB294381	UGT78F1		Not detected
T128 (S27D3)	AB294387	UGT73A8		THC2′GT>THC4′GT
T170*	AB294386	UGT71F3		THC2′GT
T172	AB294385	UGT76K2	ppt	Not detected
T181	AB294384	UGT84A7	ppt	Not detected
T206	AB294383	UGT76K1	ppt	Not detected
T227	AB294382	UGT85L1	Not clear	Not detected
Cyclamen				
CpYCy3-12	AB294395	UGT71E4		THC2′GT
CpYCy11	AB294396	UGT72B7		Not detected
CpYCy17	AB294397	UGT72B6	ppt	Not detected
CpYCy31	AB294398	UGT71H1	Not clear	Not detected
CpYCy87	AB294399	UGT88A2	Not clear	Not detected
CpPCy1-12	AB294400	UGT71E3		Not detected
Catharanthus				
CrYMb4	AB294401	UGT71E2		THC2′GT

* The GTs exhibited THC2′GT activity in the petunia petals. ppt: an expressed protein band was observed only in the precipitant fraction; not clear: no significant band on the SDS-PAGE gel was observed.

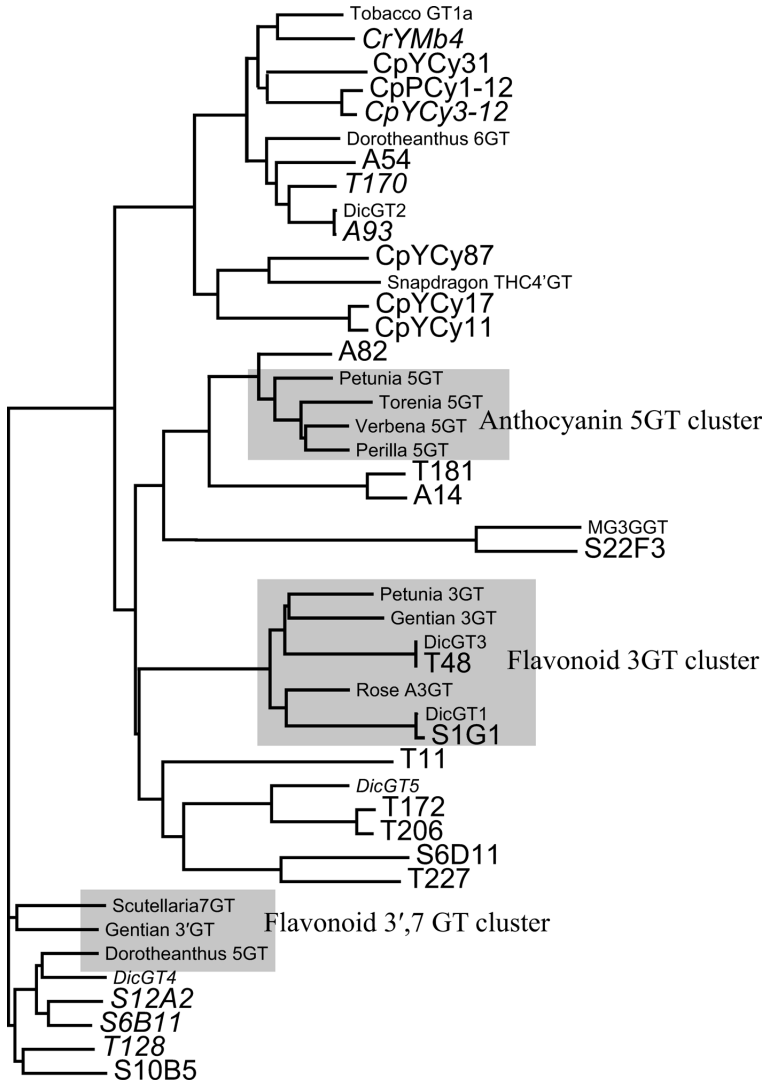

Figure 2. Non-rooted phylogenetic tree of putative flavonoid or betalain-related glucosyltransferases. The tree was constructed on the basis of a previous report (Fukuchi-Mizutani et al. 2003). The amino acid sequences of these GTs were aligned using CLUSTALW program (available at DDBJ http://clustalw.ddbj.nig.ac.jp/top-e.html) and the tree was constructed by TREEVIEW (Page 1996). The sequences isolated in this study are shown in bigger letters, and those having THC2′GT or THC4′GT activity are italicized. The accession numbers for the sequences are shown in parentheses: Flavonoid 3GT cluster members (Petunia 3GT (AB027454), DicGT3 (AB191247), Gentian 3GT (Q96493), DicGT1 (AB191245), Rose anthocyanidin 3GT (BAF80946)); anthocyanin 5GT cluster members (Petunia 5GT (BAA89009), Torenia 5GT (AB076698), Verbena 5GT (BAA36423), Perilla GT (AB013596)); 3′,7GT cluster members (*Scutellaria baicalensis* flavonoid 7GT (BAA83484), Gentian 3′GT (BAC54092), *Dorotheanthus* betanidin 5GT (CAB56231), DicGT4 (AB191248); Tobacco GT1a (BAB60720), *Dorotheanthus* betanidin 6GT (AAL57240), Snapdragon THC4′GT(BAE48239); Morning glory 3GGT (BAD95883), DicGT5 (AB191248).

Measurement of THCGT activity of the recombinant GT expressed by E. coli

The amount of recombinant proteins in the soluble fraction estimated by the intensity of the stained protein bands varied to a large extent. Some recombinant GT proteins (S10B5, S22F3, T11, T172, T181, T206, CpYCy17) are detected only in the precipitant fraction (Table 1). Recombinant T227, CpYCy31, and CpYCy87 protein bands were not observed in either supernatant or precipitant fractions (Table 1). We thus assumed that these genes are unlikely to encode THC2′GT activity since we could detect anthocyanidin 3-GT and anthocyanin 3′GT activities (Tanaka et al. 1996 and Fukuchi-Misutani et al. 2003, respectively) even when the expressed protein bands were not observed in SDS-PAGE. Further studies are necessary to identify their function(s).

The results of the THCGT assay of the recombinant GT are summarized in Table 1. The HPLC elution profiles derived from the enzymatic assays are presented in Figure 3. Recombinant A93, T128, T170, S12A2, S6B11, CpYCy3-12, and CrYMb4 displayed THC2′GT activity, and among these, T128 also exhibited additional THC4′GT activity. S6B11 displayed higher THC4′GT

than THC2′GT activity. The results of the enzymatic assay are also summarized in Table 1. Since carnations do not accumulate THC 4′-glucoside, THC4′GT activity detected in this study may not be physiological.

Expression of THCGT cDNAs in petunia

Binary vectors containing the cDNAs of *A93*, *T128*, *T170*, *S12A2*, *S6B11*, *CpYCy3-12*, and *CrYMb4* that encoded THC2′GT activity *in vitro* were constructed and introduced to the two petunia lines. The T-DNA region structures are schematically presented in Figure 4. Twenty to 30 independent transgenic plants were generated, and the lines that strongly expressed *GT* genes were selected on the basis of amplified DNA bands by 25 RT-PCR cycles. The *CHI* transcripts decreased in the petals of some transgenic petunia harboring these T-DNAs (data not shown), but no change in petal color was observed. Flowers of some transgenic lines transcribing double strand *F3H* gene exhibited white or paler flower color than the hosts, which is due to the decrease of *F3H* transcripts and the accompanying downregulation of anthocyanin biosynthesis (data not shown). However, yellow colored flowers were not obtained.

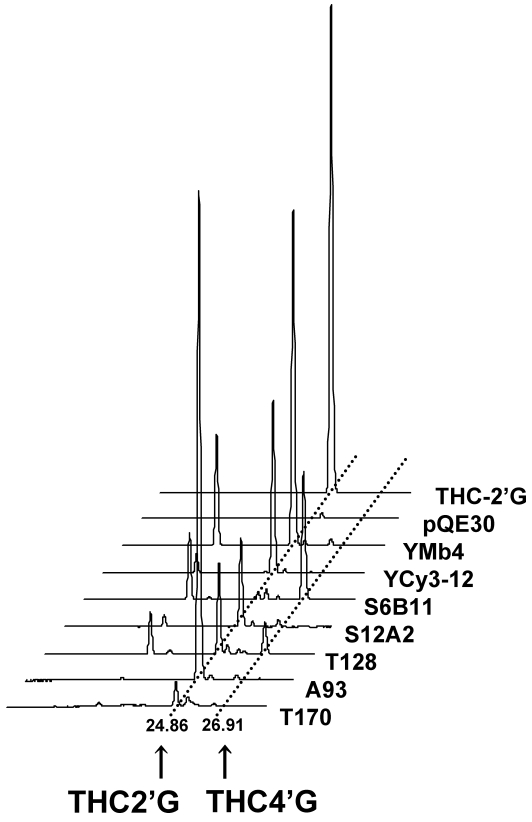

Figure 3. HPLC profiles of THCGT activity encoded by *GT* genes expressed in *E. coli*. A peak eluted at around 20 min by T128, S6B11, YCy3-12, and YMb4 had a flavanone-like absorption spectrum rather than chalcone, and is likely to be naringenin glucoside.

Representative HPLC profiles of the flavonoids of transgenic plant petals are shown in Figure 5. THC 2′ or 4′-glucoside was not found in any of the hosts (Figure 5B and 5D). Small amounts of THC 2′-glucosides were detected in some of the transgenic petunia petals (Skr4×SW63) with *S6B11* cDNA expression and downregulated *CHI* gene (data not shown); Skr4×SW63 with *S6B11*, and downregulated *CHI* and *F3H* (Figure 5C); Baccara Red with *A93*, and downregulated *CHI* and *F3H* (Figure 5E); Skr4×SW63 with *T170*, and downregulated *CHI* and *F3H* (data not shown); Baccara Red with *T170*, and downregulated *CHI* and *F3H* (Figure 5F); and Skr4×SW63 with *T170*, and downregulated *CHI* (data not shown). THC 2′-glucoside was not detected in Skr4×SW63 with *A93* cDNA, and downregulated *CHI* and *F3H*. *S6B11* cDNA was not introduced to Baccara Red. These results showed that at least *S6B11, A93*, and *T170* cDNA encoded THC2′GT activity *in vivo*. It is intriguing that S6B11 exhibited stronger THC4′GT activity than THC2′GT *in vitro* (Figure 3), and only THC2′GT activity was detected in

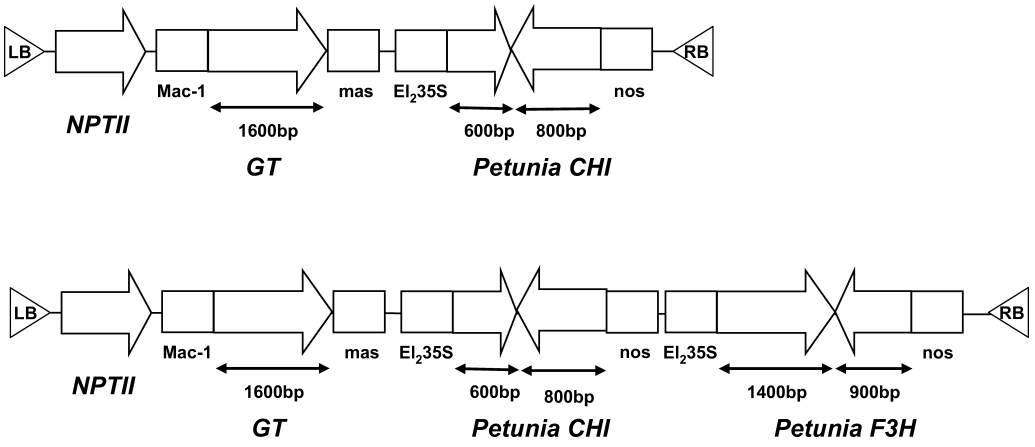

Figure 4. T-DNA structures of the binary vectors constructed for the expression of a *GT* gene and the downregulation of anthocyanin biosynthesis. Double-stranded RNA of petunia *CHI* gene or those of petunia *CHI* and *F3H* genes were transcribed in these vectors. LB, left border; nptII, neomycine phosphotransferase; Mac-1, Mac-1 promoter (Comai et al. 1990); mas, manopine synthase terminator; El235S, El235S promoter (Mitsuhara et al. 1996); nos, nopaline synthase terminator; RB, right border.

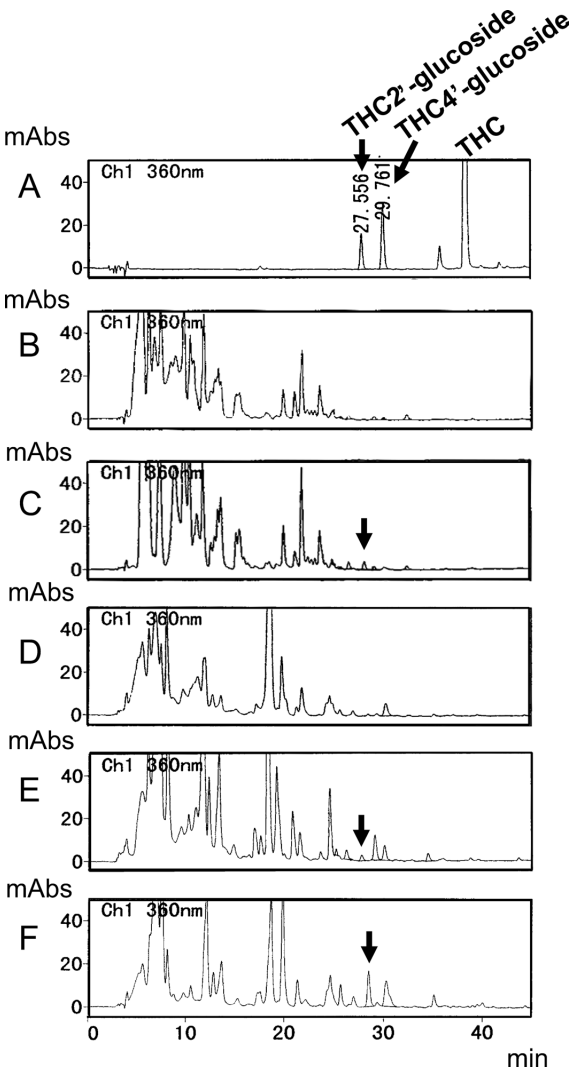

Figure 5. HPLC profiles measured at 360 nm. (A) Standard compounds. THC2′-glucoside and THC4′-glucoside were eluted at about 27.55 min and 29.76 min, respectively. (B) Skr4×Sw63, (C) Skr4×Sw63 expressing *S6B11* cDNA, (D) Bacarra Red, (E) Bacarra Red expressing *A93* cDNA, (F) Bacarra Red expressing *T170* cDNA. The transgenic plants contained small amounts of THC2′-glucoside (arrow). THC2′-glucoside was identified by its retention time and absorption spectrum as measured using the photo diode array.

transgenic petunia (Figure 5C). On the other hand, THC2′GT activity was not detected in the petunia plants expressing *A128*, *S12A2*, *CpYCy3-12*, and *CrYMb4* genes. These genes may not encode THC2′GT activity. It is also possible that efficient isomerization of THC masked their THC2′GT activity.

It is interesting that carnation contains plural GTs exhibiting THC2′GT activity. Here we additionally isolated GTs other than *DicGT4* and *DicGT5*, which have been isolated previously (Ogata et al. 2004). These GTs catalyzing THC glucosylation belong to several clusters (Figure 2). It is possible that more than one GT catalyze THC2′-glucosylation *in vivo*, and that such GT activity becomes evident only when *CHI* gene function is disrupted, as has been reported in carnations (Itoh et al. 2002) and cyclamens (Miyajima et al. 1991). It is also possible that the THC2′GT activity detected in this study is not the physiological activity of GTs. Some GTs have broad substrate specificity; for example, *Dorotheanthus bellidiformis* betanidin 5GT catalyzes glucosylation of flavonoid 4′ and 7-glucosylation in addition to betanidin 5-glucosylation (Vogt et al. 1999). Color changes from yellow to white by downregulation of *GT* genes exhibiting THC2′GT activity in carnations, cyclamens, or catharanthus may lead to the identification of physiological *THC2′GT* genes. However, transformation of these plants is difficult.

The expression of three *GT* cDNAs (*S6B11*, *A93*, and *T170*) resulted in THC 2′-glucoside in transgenic petunia (Figure 5). These *GT* genes may be useful molecular tools for engineering yellow flowers, although the amount of accumulated THC 2′-glucoside was too small to confer yellow hue to flower color in this study. Elevation of the expression level of the *GT* genes and complete suppression of CHI activity may lead to the accumulation of THC2′-glucoside. Downregulation of *CHI* gene and downregulation of anthocyanin biosynthesis have been achieved in tobacco (Nishihara et al. 2005). Introduction of *THC2′GT* genes in this study to such tobacco may confer visible yellow color.

Acknowledgements

This work was supported by the Bio-oriented Technology Research Advancement Institution. The authors thank Drs. Ludwig and Ohashi for providing *A. tumefaciens* Agl0 and pBE2113-GUS, respectively. The authors are also grateful to Dr. Mackenzie for glucosyltransferase nomenclature.

References

Comai L, Moran P, Maslyar D (1990) Novel and useful properties of a chimeric plant promoter combining CaMV35S and MAS elements. *Plant Mol Biol* 15: 373–381

Davies KM, Bloor SJ, Spiller GB, Deroles SC (1998) Production of yellow colour in flowers: redirection of flavonoid biosynthesis in Petunia. *Plant J* 13: 259–266

Fukuchi-Mizutani M, Okuhara H, Fukui Y, Nakao M, Katsumoto Y, Yonekura-Sakakibra K, Kusumi T, Hase T, Tanaka Y (2003) Biochemical and molecular characterization of a novel UDP-glucose:anthocyanin 3′-*O*-glucosyltransferase, a key enzyme for blue anthocyanin biosynthesis, from gentian. *Plant Physiol* 132: 1652–1663

Gachon CM, Langlois-Meurine M, Sainrenan P (2005) Plant secondary metabolism glycosyltransferases: the emerging functional analysis. *Trends Plant Sci* 10: 542–549

Hirotani M, Kuroda R, Suzuki H, Yoshikawa T (2000) Cloning and expression of UDP-glucose:flavonoid 7-*O*-glucosyltransferase from hairy root cultures of *Scutellaria baicalensis*. *Planta* 210: 1006–1013

Holton TA, Brugliera, F, Lester, DR, Tanaka Y, Hyland CD, Menting JGT, Lu C-Y, Farcy E, Stevenson TW, Cornish EC

(1993) Cloning and expression of cytochrome P450 genes controlling flower colour. *Nature* 366: 276–279

Itoh Y, Higeta D, Suzuki A, Yoshida H, Ozeki Y (2002) Excision of transposable elements from the chalcone isomerase and dihydroflavonol 4-reductase genes may contribute to the variegation of the yellow-flowered carnation (*Dianthus caryophyllus*). *Plant Cell Physiol* 43: 578–585

Katsumoto Y, Mizutani M, Fukui Y, Brugliera F, Holton T, Karan M, Nakamura N, Yonekura-Sakakibara K, Togami J, Pigeaire A, et al. (2007) Engineering of the rose flavonoid biosynthetic pathway successfully generated blue-hued flowers accumulating delphinidin. *Plant Cell Physiol* 48: 1589–1600

Kuhn B, Forkman G, Seyffert W (1978) Genetic control of chalcone-flavanone isomerase activity in *Callistephus chinensis*. *Planta* 138: 199–203

Lazo G, Pascal A, Ludwig R (1991) A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. *Bio/Technol* 9: 963–967

Matsuba Y, Sasaki N, Tera M, Okumura M, Abe Y, Okamoto E, Nakamura H, Funabashi H, Takatsu M, Saito M, et al. (2010) A novel glucosylation reaction on anthocyanins catalyzed by acyl-glucose-dependent glucosyltransferase in the petals of carnation and delphinium. *Plant Cell* 22: 3374–3389

Mitsuhara I, Ugaki M, Hirochika H, Ohshima M, Murakami T, Gotoh Y, Katayose Y, Nakamura S, Honkura R, Nishimiya S et al. (1996) Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. *Plant Cell Physiol* 37: 49–59

Miyajima I, Maehara T, Kage T, Fujieda K (1991) Identification of the main agent causing yellow color of yellow-flowered cyclamen mutant. *J Jpn Soc Hort Sci* 60: 409–414

Mizukami Y, Fukuta S, Kanbe M (2004) Production of yellow flower cyclamen through *Agrobacterium tumefaciens* mediated transformation with chalcone reductase. *Res Bull Aichi Agri Res Ctr* 36: 59–63

Morita Y, Hoshino A, Kikuchi Y, Okuhara H, Ono E, Tanaka Y, Fukui Y, Saito N, Nitasaka E, Noguchi H, et al. (2005) Japanese morning glory dusky mutants displaying reddish-brown or purplish-gray flowers are deficient in a novel glycosylation enzyme for anthocyanin biosynthesis, UDP-glucose:anthocyanidin 3-*O*-glucoside-2″-*O*-glucosyltransferase, due to 4-bp insertions in the gene. *Plant J* 42: 353–363

Nakayama T, Yonekura-Sakakibara K, Sato T, Kikuchi S, Fukui Y, Fukuchi-Mizutani M, Ueda T, Nakao M, Tanaka Y, Kusumi T, et al. (2000) Aureusidin synthase: A polyphenol oxidase homolog responsible for flower coloration. *Science* 290: 1163–1166

Nishihara M, Nakatuska T (2010) Genetic engineering of novel flower colors in floricultural plants: Recent advances via transgenic approaches. In: *Protocols for in vitro propagation of ornamental plants.* Jain SM, Ochatt SJ (eds.) Humana Press, Totowa, New Jersey, pp 325–347

Nishihara M, Nakatsuka T, Yamamura S (2005) Flavonoid components and flower color change in transgenic tobacco plants by suppression of chalcone isomerase gene. *FEBS Lett* 579: 6074–6078

Ogata J, Itoh Y, Ishida M, Yoshida H, Ozeki Y (2004) Cloning and heterologous expression of a cDNA encoding flavonoid glucosyltransferases from *Dianthus caryophyllus*. *Plant Biotechnol* 21: 367–375

Ono E, Fukuchi-Mizutani M, Nakamura N, Fukui Y, Yonekura-Sakakibara K, Yamaguchi M, Nakayama T, Tanaka T, Kusumi T, Tanaka Y (2006) Yellow flowers generated by expression of the aurone biosynthetic pathway. *Proc Natl Acad Sci USA* 103: 11075–11080

Page RDM (1996) An application to display phylogenetic trees on personal computers. *Comput Appl Biosci* 12: 357–358

Tanaka Y, Brugliera F, Chandler S (2009) Recent progress of flower colour modification by biotechcnology. *Int J Mol Sci* 10: 5350–5369

Tanaka Y, Brugliera F, Kalc G, Senior M, Dyson B, Nakamura N, Katsumoto Y, Chandler S (2010) Flower colour modification by engineering the flavonoid biosynthetic pathway: practical perspectives. *Biosci Biotechnol Biochem* 74: 1760–1769

Tanaka Y, Katsumoto Y, Brugliera F, Mason J (2005) Genetic engineering in floriculture. *Plant Cell Tissue Organ Cult* 80: 1–24

Tanaka Y, Sasaki N, Ohmiya A (2008) Plant pigments for coloration: Anthocyanins, betalains and carotenoids. *Plant J* 54: 733–749

Tanaka Y, Yonekura K, Fukuchi-Mizutani M, Fujiwara H, Ashikari T, Kusumi T (1996) Molecular and biochemical characterization of three anthocyanin synthetic enzymes from *Gentiana triflora*. *Plant Cell Physiol* 37: 711–716

van Engelen FA, Molthoff JW, Conner AJ, Nap J, Pereira A, Stiekema WJ (1995) pBINPLUS: An improved plant transformation vector based on pBIN19. *Transgenic Res* 4: 288–290

Vogt T, Grimm R, Strack D (1999) Cloning and expression of a cDNA encoding betanidin 5-*O*-glucosyltransferase, a betanidin- and flavonoid-specific enzyme with high homology to inducible glucosyltranferases from the Solanaceae. *Plant J* 19: 509–519

Yamazaki M, Gong Z, Fukuchi-Mizutani M, Fukui Y, Tanaka Y, Kusumi T, Saito K (1999) Molecular cloning and biochemical characterization of a novel anthocyanin 5-*O*-glucosyltransferase by mRNA differential display for plant forms regarding anthocyanin. *J Biol Chem* 274: 7405–7411

Yonekura-Sakakibara K (2009) Functional genomics of family 1 glycosyltransferases in *Arabidopsis*. *Plant Biotechnol* 26: 267–274